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R. Howard Berg • Christopher G. Taylor
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Cell Biology of Plant Nematode Parasitism

Springer

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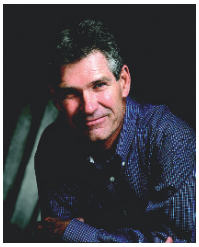

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Preface

Parasites, cysts and root-knot nematodes have evolved sophisticated mechanisms for exploiting plants, with profound agricultural impact. The susceptibility of plants to nematode parasitism has resulted in a significant effort to identify the cellular and molecular mechanisms involved in nematode-induced pathology of plants. We have been fortunate to gather a group of leading scientists who present in this book the current knowledge on nematode parasitism. Plant-nematode interactions are examined from organismal responses down to molecule-specific responses within the nematode and its host plant. In this exciting era of cell biology, computer-enhanced technology, ranging from microscopy to genomic analysis, is bringing us ever closer to using the knowledge generated to reduce the parasitic effects of nematodes on plants.

This book will be a useful reference for advanced undergraduate, graduate and postdoctoral students, as well as senior scientists.

We gratefully acknowledge the help of a number of people in reviewing and editing the manuscript, including Christine Ehret and Marti Shafer of the Danforth Plant Science Center and Jim McCarter, Michelle Hresko, and Bingli Gao of Divergence, Inc.

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Plant Infection by Root-Knot Nematode

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Abstract Plant-parasitic nematodes, particularly the sedentary endoparasitic forms, are cosmopolitan pests, collectively causing over $100 billion in annual crop loss worldwide. In the past decade, significant progress has been made in identifying genes and their products that define key aspects of the host–parasite interface, including enzymes and proteins with direct roles in virulence and resistance. However, little remains known about how a host is identified or how the development of the nematode is coupled to establishment of the parasitic interaction. Here, we consider the role of signaling molecules and their interplay with nematode development from hatch through primary interaction with the plant.

1 A Brief Introduction to Root-Knot Nematode

Although plant-parasitic nematodes are found in three of the five major clades of the phylum Nematoda (Blaxter et al. 1998), much of the damage to crops is caused by the approximately 60-member tylenchid genus, *Meloidogyne* (Sasser and Freckman 1986; Koenning et al. 1999). Reflecting the gross symptoms exhibited by roots infected with these nematodes (Fig. 1), the common name for *Meloidogyne* spp. is "root-knot nematode(s)." More than 2,000 plant species have been designated as hosts to root-knot nematodes, and most cultivated crops are attacked by at least one root-knot nematode species (Sasser 1980). Since its description as a genus (Chitwood 1949), root-knot nematodes have been particularly favored for research by plant nematologists in large measure because of their importance as agricultural parasites. Beyond this, however, the motivation to study root-knot nematode has sprung from scientific curiosity regarding the many intriguing features of their

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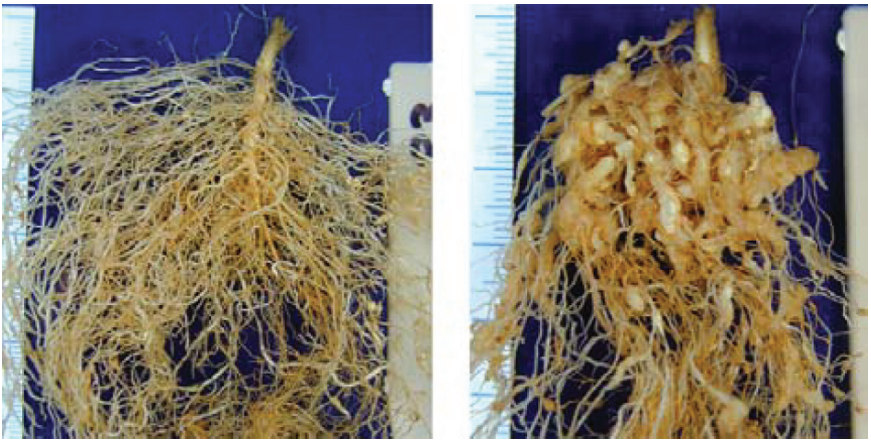

Fig. 1 Symptoms of root-knot nematode infection. Root systems of *Medicago truncatula* plants inoculated with *Meloidogyne incognita*. The plant on the *left* carries a gene conferring resistance to *M. incognita*, whereas the plant on the *right* is susceptible. Characteristic root knots (galls) are evident on the roots of the infected plant

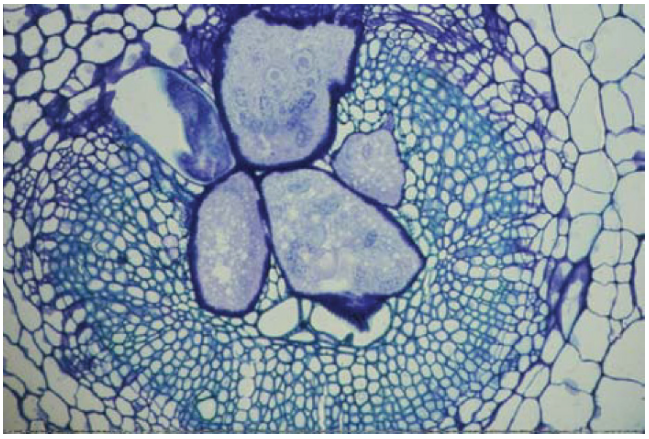

Fig. 2 Transverse section through a mature root gall induced by *Meloidogyne incognita* in tomato, stained with toluidine blue. Four giant cells are evident in the center of the vascular cylinder, surrounded by numerous small cells. The head of the nematode has contracted during fixation, leaving a partially hollow space adjacent to the giant cells

parasitic lifestyle, the most striking of which is the induction of so-called "giant cells" in the host root vasculature (Fig. 2). Induction of giant cells uniquely defines the *Meloidogyne*–host interaction and is central to it because these cells apparently serve as the sole food source for the developing worm.

Very briefly, root-knot nematodes hatch in the soil as motile, vermiform larvae (Fig. 3) able to locate, penetrate and migrate within plant roots (Fig. 4), ultimately

Fig. 3 Newly hatched *Meloidogyne* J2. *Arrows* point to some of the numerous lipid storage vesicles throughout the nematode's body, and the *bar* (S) indicates the retracted stylet

Fig. 4 Root-knot nematode J2 migrating through cleared *Lotus japonicus* roots. The worms were stained with acid fuschin

reaching the developing vascular cylinder where the giant cells are established. Giant cell formation, coupled with expansion and proliferation of nearby pericycle and cortical cells, results in the characteristic root-knot gall. Like many other root-colonizing organisms, root-knot nematodes reside in the apoplast once inside the plant, obtaining nutrition from the symplast through an as yet poorly understood process. Mature females lay eggs out into the soil to complete the lifecycle.

This chapter focuses on the biological events that lead the root-knot nematode to its selection of a host and the irrevocable commitment by the parasite to a sedentary lifestyle. In other words, we consider the events that occur between hatch and the first meal, ending our discussion prior to giant cell ontogeny and operation (Gheysen and Mitchum 2008; Berg et al. 2008). Our focus is on the nematode rather than the host, although in reality both must play equally in the host–parasite interaction.

Our intent is twofold. First, we will discuss events that take place prior to root penetration, arguing that nematode behavior reflects responses to multiple environmental signals. Because little is known yet about the nature of such signals, this will be a short section by necessity. One considerable impediment to progress stems from the fact that the biology prior to host penetration occurs within the complex four-dimensional milieu that is the rhizosphere and surrounding soil. Although some studies attempted to make direct observations of nematodes in the soil (e.g., Pitcher 1967), most of our current understanding comes from analysis of in vitro systems. In the soil, the host for the root-knot nematode is very literally a "moving

target" that is not well modeled in vitro. Similarly, once the nematode has penetrated its host, direct experimental manipulation becomes extremely difficult. Not surprisingly, most of what is known of the biological events associated with root-penetration and subsequent migration comes either from destructive analysis (e.g., following fixation and staining) or from inference based on in vitro experiments.

For at least 40 years (e.g., Bird 1964), a particular focus has been on the proteins secreted by the root-knot nematode second-stage juvenile (J2) during and after migration through the root, and a picture is emerging of the myriad roles played by these proteins (e.g., Baum et al. 2007). We will make no further mention of these proteins in this chapter, not to diminish their importance, but because they are discussed in detail elsewhere in this volume (Davis et al. 2008). Our second goal, therefore, is not to describe the "machinery" deployed by the J2, but rather to frame the events that lead to host selection as a behavioral response by the nematode that culminates in an irrevocable developmental commitment. Despite only a limited data set in this area, a picture is emerging of complex communication between host and parasite that likely influences the behavior of both. Additional signaling between other environmental components, including other rhizosphere organisms, contributes to the complexity. Deciphering these networks may be an important step towards truly understanding plant infection by root-knot nematode.

2 The Root-Knot Nematode Larva at Hatch

Like all nematodes, root-knot nematode embryogenesis/morphogenesis occurs within an environmentally resilient egg, whose shell is principally composed of protein (50%), chitin (30%), and lipid (Bird and McClure 1976). The egg is the most robust life stage of the nematode and precludes passage of even small molecules (such as the fungal toxin α-amanitin) that readily penetrate the cuticle of hatched stages (Rogalski and Riddle 1988). Rendering the egg sensitive to α-amanitin requires the drastic treatment of chitinase digestion followed by mechanical stripping of the vitelline membrane (Edgar et al. 1994). Thus, for root-knot nematodes there is no evidence of the developing larvae perceiving external clues, but it is not inconceivable that such events may take place. Indeed, other tylenchid nematodes, particularly *Globodera* species, almost completely depend on perception of a host-derived signal to induce substantial hatch. On the basis of purification from potato root diffusate, one component of the hatch signal has been proposed to be *trans*-2-(2,13-dihydroxy-9-methoxy-7,7,16-trimethyl-5,10,20-trioxo-19-oxahexacyclo[9.7.0.1^{3},6.0^{3},8.1^{12},15.0^{12},16]-eicosa-1(11),8-dien-15-yl) cyclopropanecarboxylic acid (Mulder et al. 1996).

Immediately prior to hatch, the root-knot nematode eggshell undergoes structural transformation, rendering it permeable to a number of reagents, such as the electron microscopy fixative/stain osmium tetroxide, to which younger eggs are resistant (e.g., Fig. 5 in Bird and Bird 1991). Unlike most nematodes, root-knot nematodes undergo the first of their larval four molts within the egg, thus hatching

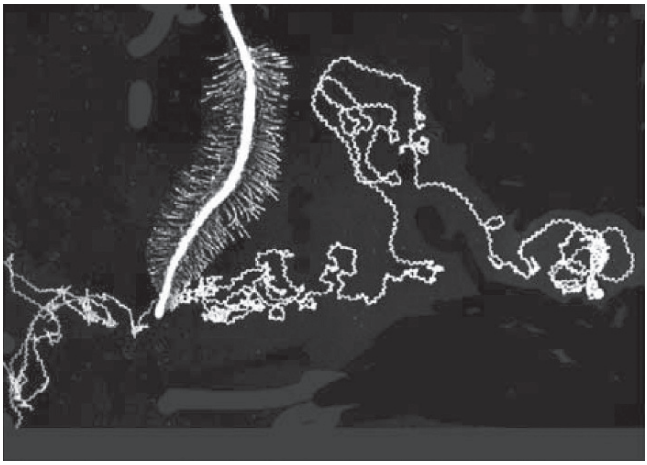

Fig. 5 Root-knot nematode attraction to root. Darkfield image of the track left in an agar surface by two root-knot nematode individuals as they migrated to the root tip

as a J2. Very little is known about the relative importance of eggs and J2s in dormant periods of root-knot nematode such as over wintering or between hosts; however, it is likely that the J2 is the predominant dormant stage because hatch does not require an external cue. Indeed, numerous lines of evidence point to the root-knot nematode (and other tylenchid nematodes) J2 as being analogous to the dauer ("enduring") stage of *Caenorhabditis elegans* (Riddle and Georgi 1990; Bird and Opperman 1998; Opperman and Bird 1998). The dauer was first described as an adaptation by animal-parasitic nematodes (Fuchs 1915), but subsequently appreciated as a phylum-wide phenomenon, extending to plant-associated genera as well (e.g., Fuchs 1937; Bird and Buttrose 1974). Dauers share the properties of arrested development, motility, non-feeding, non-ageing and hence longevity (Cassada and Russell 1975; Klass and Hirsh 1976; Riddle and Albert 1997), attributes that accurately describe root-knot nematode J2s. *C. elegans* dauers also exhibit characteristic morphological features, such as sparse (compared to L3) luminal microvilli, numerous lipid storage vesicles, and a denser cuticle that results in elevated detergent resistance; these features all have been found in tylenchid J2s (Endo 1988; Opperman and Bird 1998) (Fig. 3). A consequence of the suspension of ageing by the root-knot nematode dauer is that the time spent as a J2 largely determines the egg-to-egg time for any given individual.

Dauers have been most extensively studied in *C. elegans*, where they function as facultative, alternative stage-three larvae (L3) and serve as a binary switch to broadly couple larval development to sexual maturity with "boom" (L3) or "bust" (dauer) conditions. On the basis of an elegant amalgam of genetic, biochemical and developmental experiments (reviewed by Riddle and Albert 1997), it was shown that the stage-one larvae (L1) integrates the environmental cues of "dauer-pheromone" and "food signal" (and, to a lesser extent, temperature) to instruct the stage-two larvae (L2) development and the product of the L2 molt (Golden and Riddle 1982).

Dauer-pheromone, which has recently been attributed to three related ascarosides, viz., (-)-6-(3,5-dihydroxy-6-methyltetrahydropyran-2-yloxy) heptanoic acid, 5-O-ascarylosyl-5R-hydroxy-2-hexanone, and an ascaroside derivative of 8R-hydroxy-2E-nonenoic acid (Jeong et al. 2005; Butcher et al. 2007), is constitutively expressed by *C. elegans* and serves as a "quorum sensing" vehicle by which each individual L1 assesses population density. The precise nature of the "food signal" remains obscure but is employed as the numerator for the pheromone denominator; a relative value of >1 says "boom," whereas <1 says "bust."

Unlike in *C. elegans*, the root-knot nematode dauer (J2) stage is obligate. Whether this points to elimination of the inductive pheromone in root-knot nematodes is unknown although, intriguingly, the adaptation of undergoing the first molt within the egg would appear to ensure that the assessed food:pheromone ratio would always be <1. Another difference between *C. elegans* and root-knot nematode dauers is the developmental stage at which they occur (L3 in the former, J2 in the latter). However, as previously discussed (Bird and Opperman 1998), such heterochronic shifts are common across evolutionary space. Indeed, the dauers of *Bursaphelenchus* occur in stage-four larvae (Fuchs 1937). Although any potential role of dauer pheromone in root-knot nematode development is arcane, a role for the food signal seems clear; dauer exit, which involves the simultaneous resumption of development and ageing and a switch in carbon source from internal (i.e., stored lipid) to external, is strictly coupled to the onset of feeding inside the selected host plant (Bird 1996).

Direct biochemical analyses of intermediary metabolism (O'Riordan and Burnell 1989; Wadsworth and Riddle 1989; O'Riordan and Burnell 1990) have revealed that the *C. elegans* dauer larva is metabolically distinct from other stages, presumably reflecting the importance of lipid metabolism for this non-feeding stage. On the basis of the assumption that this biology is likely to be conserved across the phylum, approaches based on data mining (Mitreva et al. 2004) and microarray analysis (Elling et al. 2007) have attempted to compare the dauer transcriptome of the animal-parasitic nematode *Strongyloides stercoralis* and the plant parasite *Heterodera glycines*, respectively, with that of the *C. elegans* dauer. With the strong twin caveats that in each of these experiments (1) only a limited subset of the parasite transcriptome was sampled, and (2) only steady-state dauers were sampled (i.e., the transcriptome was not sampled during dauer entry or exit), both groups concluded that there is no clear evidence for a conserved dauer gene-expression signature across the Nematoda. If this conclusion is correct, it presumably reflects the unique biological adaptations of these diverse nematode species. Whether the same conclusion would be drawn from an experiment comparing the root-knot nematode transcriptome during dauer recovery with gene expression during dauer exit in *C. elegans* (Jones et al. 2001) remains untested.

In contrast to the transcriptome experiments, evidence is accumulating to support the hypothesis that the dauer pathway per se is utilized to regulate dauer entry and exit across the phylum (Blaxter and Bird 1997; Bürglin et al. 1998; Bird and Opperman 1998; Bird et al. 1999). Considerable functional evidence from *C. elegans* points to the dauer pathway as being primarily neuronally mediated, beginning with

perception of the primary effectors (pheromone and food) by the amphids and presumably transmitted downstream by endocrine function. Genetic analysis in *C. elegans* identified 32 genes as dauer affecting (*daf*) (Riddle and Albert 1997), and the molecular nature of 20 of these has been discerned, revealing a signaling pathway that is highly conserved across the animal kingdom, including humans (Wolcow et al. 2002), and which assesses nutrient status and allocates energy resources to development, ageing and fat deposition (i.e., collectively the core of dauer function). *C. briggsae* encodes 19 of the 20 characterized *C. elegans* daf genes but lacks *daf-28*, which encodes the beta-insulin molecule involved in signal transduction. Recent whole genome sequencing of *M. hapla* (Opperman et al. in press; Opperman et al. 2008) has revealed strong orthologs of 14 *C. elegans daf* genes and weak orthologs of three more. Like *C. briggsae*, *M. hapla* lacks an ortholog of *daf-28*. The molecular identities of those genes not found in *M. hapla* appear related to perception of specific cues and, hence, are probably not relevant to the parasitic lifestyle of root-knot nematodes. This demonstrates that, although the basic mechanical aspects of development are conserved, response to environment in parasite versus free-living nematode is substantially distinct, consistent with the transcriptome results (Mitreva et al. 2004; Elling et al. 2007).

3 From Hatch to Root-Penetration

Behaviorally, newly hatched root-knot nematode J2s display random movement when no attractants are present but switch to oriented migration through a concentration gradient and toward a host root tip (Perry and Aumann 1998) (Fig. 5). Although nematode movement is characterized by sinusoidal movements, migrating root-knot nematodes often exhibit quite sharp bends (Fig. 5). Plant-parasitic nematodes are attracted to host roots presumably due to a concentration gradient of substances from the root (Bird 1959; Riddle and Bird 1985), but little is known about the identity of the substances forming these gradients. There are also differences in attraction depending on plant species, the conditions of the assay and, for example, the presence or absence of border cells (Zhao et al. 2000). Although various salts and chemicals have been reported to be attractants, many of these findings have not been substantiated. However, both plant parasitic and free-living nematodes show chemotaxis along gradients of carbon dioxide (Dusenbery 1983; Robinson 1995). Once the J2s reach the root, they accumulate at the zone of elongation. Marked changes in nematode behavior ensue and are characterized by stylet thrusting, probing, and other activities associated with root penetration (Wyss et al. 1992). Aggregation of juveniles occurs, possibly indicating communication between worms by a pheromone (Fig. 6).

Analysis of the cell biology of the response of the host root surface to root-knot nematode exposure also reveals a complex but subtle interaction (Weerasinghe et al. 2005). Briefly, exposure of J2s that have not been exposed to roots since hatching rapidly elicits a wavy root-hair response on the roots of various plant

Fig. 6 Aggregation of root-knot nematodes at the root surface. *Meloidogyne hapla* J2 permitted to migrate to a tomato root in a gel assay accumulate in large masses on the root surface at the zone of elongation. Photos by C. Wang

species tested (including tomato and *Lotus japonicus*). However, repeating this experiment using J2s that have been previously exposed to the host, even for only a brief period (several minutes), elicits striking developmental changes in the root-hair cells, including rapid ionic fluxes, cytoskeletal reorganization, and nuclear relocation (Weerasinghe et al. 2005). Genetic analysis revealed that these host responses require components of the rhizobial Nod-factor perception pathway, with certain mutations in the Nod factor receptor kinases also reducing root-knot nematode infection by tenfold (Weerasinghe et al. 2005). Thus, it appears that components of the host signaling machinery necessary for establishment of mutualistic symbioses are also utilized for the establishment of parasitic symbioses (Lohar and Bird 2003; Bird 2004; Weerasinghe et al. 2005). One hypothesis that stems from this revelation is that evolution of parasitism in root-knot nematodes was accompanied by conscription of the older symbiotic pathways in the plant as a means to enhance the nematode's parasitic ability. Root-knot nematode exudates have also been shown to affect the shape of pea border cells (Zhao et al. 2000).

Collectively, these data also implicate the presence of a root-knot nematode-encoded signal, which has been named NemF (nematode factor). It has been hypothesized that NemF is physically more than one moiety (Weerasinghe et al. 2005). One component (responsible for wavy root-hairs) is produced by root-knot

nematodes prior to root-exposure and may be produced constitutively. Significantly, dead J2s do not produce the effect on plants, nor does the non-plant-parasite *C. elegans*. The second moiety, responsible for more profound developmental changes in the plant (including reorganization of the cytoskeleton and root-hair branching), appears to be produced only in response to the host and possibly reflects the perception by the J2 of a chemical signal from the plant.

Little is known about what changes in gene expression occur in root-knot nematodes between hatching and the onset of feeding in the host. It seems likely that the behavioral changes exhibited by the J2 in response to chemical signals from the host are accompanied by changes in gene expression, potentially including de novo transcription of genes, translation of pre-existing mRNAs, or modification of pre-existing proteins (e.g., by phosphorylation). Upon perception of a host and during movement of the non-feeding larva toward the root, it is reasonable to predict changes in expression of genes regulating metabolic activation, perhaps even similar to those genes up-regulated in *C. elegans* upon dauer exit (Jones et al. 2001). It is likely that genes more strictly involved in parasitism per se are also induced, including those necessary for production of NemF as well as yet to be identified proteins involved with host invasion and suppression of defense responses. Identification of such genes is likely to provide new insights into parasitism.

Studies on the behavior of nematodes once they have penetrated the root and during their migration to their feeding site have been difficult to study in vivo as the nematodes are hidden from view inside the roots. However, video-enhanced contrast light microscopy has been used successfully to visualize invasion of the transparent roots of *Arabidopsis* on agar plates by *M. incognita* (Wyss et al. 1992). J2s were observed destroying epidermal and sub-epidermal cells in the invasion process. This disruption was preceded by lip rubbing and stylet trusts against the cell walls. Once inside the root, the J2s migrated intercellularly between cortical and meristematic cells toward the root tip. After reaching the meristem, the J2s reversed direction, often damaging meristematic cells in the process, and then migrated upwards within the developing vascular cylinder. A feeding site is initiated near the zone of differentiation. Although caution should be used in generalizing the behavior of root-knot nematode in *Arabidopsis* to more typical hosts with more complex root structure, in vitro stained roots of other species are consistent with the same course of migration. The physical and chemical signals from the plant that contribute to the observed migration have not been determined. In addition, multiple J2s frequently invade at the same site and follow the same tract toward the vascular invasion, suggesting that signaling between nematodes may also be involved in the process.

Both mechanical force and enzyme secretions appear to be involved in host penetration and movement to feeding sites, and their relative importance is not known. Some of the numerous cell wall modifying enzymes that are secreted during the infection process are likely to be utilized as aids during invasion (reviewed elsewhere in this volume). Other secretions during invasion may be important in suppressing host defense responses. The intercellular movement, which avoids cell damage during migration to the feeding site, may also be part of the strategy to avoid host recognition as would be expected for an effective biotrophic parasite.

4 Conclusions

There is no doubt that efforts focused on understanding the mechanisms of giant cell formation will continue to be a major and important focus of plant-parasitic nematode research. But this is not the only important question. Careful analysis of the biology of the interaction strongly implies that the key events leading to successful infection by the nematode or successful defense by the host immunity are mediated by host–parasite signaling. In particular, evidence is accumulating that suggests chemical signals are passed between host and nematode prior to penetration (i.e., in the soil; Weerasinghe et al. 2005) and also between individual J2 (as aggregation or quorum sensing pheromones). It has long been appreciated that isolates of plant-parasitic nematodes differ in host range and in attraction to specific hosts, but currently there is little understanding of what host signals are recognized by the nematode and how these signals modify host behavior and gene expression. Such communication represents a particularly attractive target for chemical disruption as a strategy for nematode control as the pre-infective stage is most exposed to the environment (i.e., not protected by the egg or the plant host). A recent publication shows one path for development of novel nematicides based on genomic information about such "linchpins" of nematode biology (McCarter 2004); presumably other paths exist too.

The development of a genetic system for *M. hapla* offers another approach to identifying factors involved in host recognition and infection (Liu et al. 2007). F2 lines have been produced from strains of *M. hapla* that differ in host range and attraction to specific hosts (Liu and Williamson 2006). This resource should allow the mapping of genes that determine these phenotypes. The availability of the genome sequence of *M. hapla* together with the genetic map should lead to the eventual cloning of these traits (Opperman et al. 2008). Use of expression studies and RNAi will help in confirming gene function.

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Parasitism Genes: What They Reveal about Parasitism

Eric L. Davis(✉), R.S. Hussey, and Thomas J. Baum

Abstract Nematodes are parasites of plants and animals that have evolved diverse and often specific mechanisms to promote a given parasitic lifestyle (Baldwin et al. 2004; Jasmer et al. 2003), including modifications of developmental and reproductive potential, dissemination amongst and location of primary or alternate hosts, and survival strategies in the absence of a suitable host or favorable environment. The genetic pathways underlying these lifecycle adaptations may have parallels with or origins in nonparasitic nematode species that must also adapt to a dynamic or unstable niche. Distinct to the parasites, however, are adaptations to obtain organic nutriment while living in or on another organism. The products of such *parasitism genes* "may be manifested as morphological structures that provide access to parasitism of a particular host (e.g. a nematode stylet) or they may play critical physiological roles in the interaction of the nematode with its host" (Davis et al. 2000).

1 Introduction

The stylet (Fig. 1), a protrusible oral spear, is the primary adaptation that allows all plant-parasitic nematodes to breach the plant cell wall to access host cell nutrients, which is essential for nematode growth and reproduction (Hussey 1989). The stylet is a hardened structure of sclerotized cuticle that connects directly to the lumen of the alimentary canal in the nematode esophagus (Bird and Bird 1991), and in all but the trichodorid species, the stylet itself has a hollow lumen with an aperture that provides a continuous channel between the feeding nematode and the parasitized

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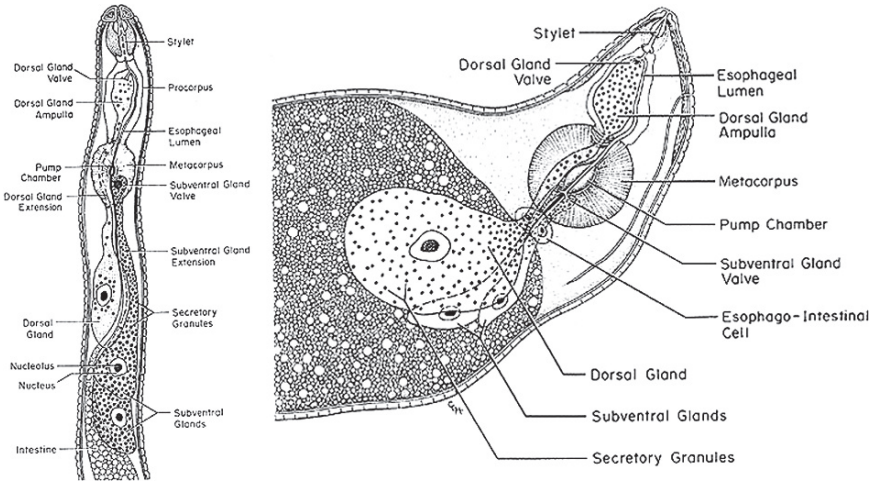

Fig. 1 Illustrations of the anterior portions of the migratory and sedentary stages of endoparasitic nematodes that contain esophageal gland secretory cells associated with the nematode stylet, a hollow oral feeding spear. (**a**) A migratory, pre-infective second-stage juvenile with the two subventral esophageal gland cells packed with secretory granules. (**b**) A swollen female from within infected roots with reduced subventral glands and an enlarged dorsal esophageal gland cell now packed with secretory granules. Reprinted from Hussey (1989) with permission from Annual Reviews

host plant cell. Successful parasitism requires feeding from a living host cell (obligate biotrophy), and for some nematode species, modifications of the host cell are required to promote a sustained feeding relationship.

Detailed investigations summarized in this volume and elsewhere indicate that molecules at the interface of the nematode and host play critical roles in the parasitic process (Hussey 1989; Davis et al. 2004, 2008; Jasmer et al. 2003; Maizels et al. 2004). Potential origins of interacting molecules from the nematode include a dynamic surface coat and natural openings such as the excretory-secretory pore, anal and reproductive openings, chemosensory organs, and the oral aperture. Since modifications of host plant cells for feeding occur at the nematode anterior, molecules secreted from the stylet and amphids emerge as the most likely organs to be directly involved in adaptations for plant parasitism. Most notably, the esophageal gland cells in tylenchid nematodes (Hussey 1989) have evolved into three relatively large secretory cells (one dorsal and two subventral) that are connected to the esophageal lumen and stylet through complex valves (Fig. 1). In the root-knot and cyst nematodes, change in the morphology, activity, and contents of the esophageal gland cells occurs throughout the course of parasitism (Davis et al. 2000, 2004). Activity within the subventral gland cells predominates in the migratory stages of these nematodes while enlargement and activity in the dorsal gland cell is dominant in the subsequent sedentary stages (Hussey 1989). The secreted protein products of parasitism genes expressed in these gland cells have been the subject of intense investigation and form a primary foundation of what we currently know about

nematode parasitism genes (Baum et al. 2006; Davis et al. 2004, 2008; Jasmer et al. 2003; Mitchum et al. 2007; Van Holme et al. 2004).

2 Parasitism Gene Discovery

Early investigations of plant-parasitic nematode secretions were biochemical in nature out of necessity and included elegant experiments that provided insights valuable to this day (Bird 1968; Hussey 1989; Veech et al. 1987). The difficulties in working with microscopic obligate parasites, especially parasitic stages from within plant tissue, remain today and provided a challenge to identify the point of origin of isolated nematode molecules in early investigations. Methods to stimulate stylet secretions from infective larvae – so-called second-stage juveniles (J2) – of the root-knot and cyst nematodes (Fig. 2) in vitro using resorcinol (McClure and von Mende 1987) and 5-methoxy DMT oxalate (Goverse et al. 1994), respectively, provided increased quantities of secretory proteins for direct analyses and antibody production. Relatively recently, secretions collected from nematode J2 stimulated in vitro were subjected to proteomic analyses, and the amino acid sequence generated from the analyses has been used to identify candidate parasitism genes (De Meutter et al. 2001; Jaubert et al. 2002). The adoption of monoclonal antibody technologies to specifically tag and isolate target nematode secretory proteins (Fig. 2) was used to identify discrete secretory proteins in plant nematodes and to monitor their differential synthesis during plant parasitism (Davis et al. 2000, 2004). An amino-terminal sequence of a cyst nematode subventral esophageal gland antigen that was affinity-purified with

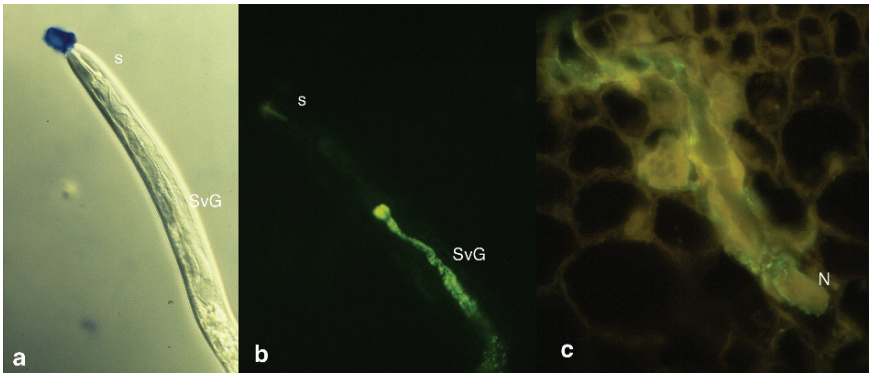

Fig. 2 Proteins produced in the nematode esophageal gland cells and secreted through the nematode stylet. (**a**) Esophageal gland proteins stained blue with Coomassie Briliant Blue are secreted from the stylet of a soybean cyst nematode (SCN) second-stage juvenile (J2) that has been incubated in the serotonin agonist, 5-methoxy-DMT oxalate. (**b**) Fluorescence immunolocalization of the antigen of monoclonal antibody MGR48 (Deboer et al. 1998) in secretory granules synthesized within the subventral esophageal gland cells of a SCN J2. (**c**) Immunolocalization of beta-1,4 endoglucanase (green fluorescence) secreted from an infective SCN J2 along its path of intracellular migration through a soybean root. Reprinted from Wang et al. (1999) with permission from APS Press

monoclonal antibody MGR48 (De Boer et al. 1996) was used to develop PCR primers to obtain the first expressed parasitism genes isolated from plant-parasitic nematodes, beta 1,4-endoglucanases (Smant et al. 1998). This discovery was able to confirm a point of origin of these cell wall-modifying enzymes as suggested in earlier investigations (Deubert and Rohde 1971). The cyst nematode cellulases were the first endoglucanase genes to be cloned from an animal and their striking similarity to bacterial Family 5 glycosyl hydrolases provided some of the first evidence of potential horizontal gene transfer from prokaryotes to eukaryotes (Smant et al. 1998; Hotopp et al. 2007; Ledger et al. 2006; Keen and Roberts 1998). A technique developed for mRNA in situ hybridization in plant nematodes (De Boer et al. 1998) and polyclonal antibodies generated to the recombinant products of the cyst nematode endoglucanase genes confirmed endoglucanase expression exclusively within the subventral esophageal glands (Smant et al. 1998). The anti-endoglucanase sera were subsequently used to confirm for the first time (Wang et al. 1999) the secretion of a nematode esophageal gland protein into plant tissues (Fig. 2).

The rapid advance of techniques in molecular biology, including methods to work with sub-microgram quantities of starting material, ushered in an era of expressed gene characterization in plant-parasitic nematodes. Complimentary DNA (cDNA) amplified from mRNA that was isolated from the dissected anterior and posterior halves of hatched, preparasitic J2 root-knot nematode, *Meloidogyne javanica*, was used to screen cDNA clones derived from the anterior halves (which contained the esophageal glands) of J2 to isolate a gene encoding a secreted chorismate mutase (CM) expressed specifically within the nematode esophageal gland cells (Lambert et al. 1999). Interestingly, the root-knot nematode CM was also most similar to genes in bacteria, and expression of *Mjcm1* in bacteria complemented a CM-deficient mutant (Lambert et al. 1999). Combined with the cyst nematode endoglucanase gene discoveries, these data encouraged early speculation that a number of plant nematode parasitism genes were derived via ancient horizontal gene transfer (Davis et al. 2000). Analyses of the genomic organization of cyst nematode endoglucanase genes have identified differences in intron size with conservation of intron position (Yan et al. 1998) and one endoglucanase gene (*Hg-eng*-5) that lacks any introns (Gao et al. 2004a). Furthermore, multiple endoglucanase genes within close genomic proximity (Yan et al. 2001) suggested the potential for "pathogenicity islands" in plant-parasitic nematodes.

The use of cDNA-AFLP to compare life stages of parasitic nematodes also has been relatively successful for the isolation of potential parasitism genes. The observation (Perry et al. 1989) that secretory granules are synthesized within the subventral esophageal gland cells of *Globodera rostochiensis* J2 within eggs upon hydration, and that subsequent exposure of the same J2 within eggs to potato root diffusate stimulated secretory granule synthesis within the dorsal gland cell (Smant et al. 1997), was exploited to compare cDNA-AFLP profiles derived from nematodes in each treatment (Qin et al. 2000). A number of differential transcript-derived fragments (TDFs) were identified among the different *G. rostochiensis* treatments, and a useful program (GenEST) was designed to cross-reference the TDFs to expressed sequences tags (ESTs) derived from cDNA libraries (Qin et al. 2001). In situ mRNA hybridization was conducted with *G. rostochiensis* clones that were differentially

expressed in cDNA-AFLP, and a number of genes expressed exclusively within the esophageal gland cells were isolated. Similar cDNA-AFLP analyses have been conducted in developmental stages of sugarbeet cyst nematodes (Tytgat et al. 2004) and among root-knot nematode near-isogenic lines (Neveu et al. 2003).

The most powerful and successful approach in identifying parasitism genes has been the direct microaspiration of the esophageal gland contents of multiple parasitic stages of *Heterodera glycines* and *Meloidogyne incognita* dissected from host roots to isolate mRNA and generate cDNA libraries that profiled esophageal gland gene expression throughout the parasitic cycle (Gao et al. 2001a, 2003; Huang et al. 2003, 2004; Wang et al. 2001). Methods of cDNA synthesis that favor inclusion of 5'-end sequence of transcripts were used to construct all gland-cell libraries. A number of cDNA selection procedures including yeast-secretion signal peptide selection (Wang et al. 2001) and subtraction against cDNA derived from nematode intestinal tissues (Gao et al. 2001a; Huang et al. 2004) have been used to identify clones within the gland cell cDNA libraries that encode secreted products that are exclusively expressed within the esophageal gland cells. Expressed sequence tag analyses have also been conducted with relatively complex gland-cell cDNA libraries that also incorporated hybridization with intestinal tissue cDNAs to gland cell macroarrays to enrich subsequent samples for unique ESTs (Gao et al. 2003; Huang et al. 2003). Putative parasitism genes were identified among gland cell cDNA clones using SignalP prediction of a putative secretion signal peptide (Nielsen et al. 1997) and confirmation of expression of the gene within the esophageal gland cells by mRNA in situ hybridization. Using these methods, more than 50 putative parasitism genes developmentally expressed in the esophageal gland cells have now been isolated in both *H. glycines* and *M. incognita*. With the exception of cell wall-modifying enzymes and a few other secreted products, relatively few common parasitism genes exist between *H. glycines* and *M. incognita* (R.S. Hussey, unpublished), and more than 70% of the parasitism gene sequences in both species have no significant database homology (i.e. so-called pioneers), indicating they may be unique to plant-parasitic nematodes.

A large-scale project designed to generate ESTs from multiple species of both plant and mammalian-parasitic nematodes had generated more than 400,000 total ESTs as of 2005 (McCarter et al. 2005; Mitreva et al. 2005a). These EST data are of tremendous significance to our understanding of nematode biology, including the potential discovery of new nematode parasitism genes. The ESTs are derived from mRNA of whole nematodes using several methods of cDNA synthesis, usually representing the life stages(s) that are most readily procured or in highest abundance. Plant-parasitic nematode ESTs (125,412) available (McCarter et al. 2005) by species include *Globodera pallida* (4,378), *Globodera rostochiensis* (5,941), *Heterodera glycines* (24,438), *Heterodera schachtii* (2,818), *Meloidogyne arenaria* (5,108), *Meloidogyne chitwoodi* (12,218), *Meloidogyne hapla* (24,452), *Meloidogyne incognita* (19,934), *Meloidogyne javanica* (7,587), *Meloidogyne paranaensis* (3,710), *Pratylenchus penetrans* (1,928), *Pratylenchus vulnus* (2,485), *Radopholus similes* (1,154), and *Xiphinema index* (9,351). The ESTs from *H. glycines* were generated from discrete, stage-specific cDNA libraries, providing a global